Rat Model of Fetal Alcohol Syndrome: Postnatal Ethanol Exposure Has Long-Lasting Effects on Adult Behavior and NMDA Receptor-Dependent Function

Caitlin DuPont

Advisor: Derick Lindquist

Abstract

Drinking alcohol while pregnant can induce neurotoxic effects during fetal development, resulting in a variety of physical and mental deficits. Symptom severity depends on the timing and peak blood alcohol concentration in both humans and animals. In our rat model of fetal alcohol syndrome (FAS), ethanol is administered across postnatal days 4-9, a period equivalent to the third trimester in humans. Adult control and FAS rats undergo trace fear conditioning (TFC), in which a neutral conditioned stimulus (CS), a tone, and an aversive unconditioned stimulus (US), a foot shock, are repeatedly paired but do not overlap in time. Following training, conditioned fear (e.g., freezing) to CS-alone presentations is reduced in FAS rats relative to control subjects. FAS rodents can, however, successfully acquire delay fear conditioning in which the CS and US overlap and co-terminate. The current study aims to determine whether the TFC deficit is specific to the interval separating the CS and US by training animals with a shorter (5 sec) or longer (15 sec) interval between stimuli. The data suggests that only FAS rats conditioned with the 15 sec trace interval display deficient freezing relative to controls. TFC relies on NMDA-receptor (NMDAR) dependent plasticity in the medial prefrontal cortex (mPFC) and dorsal hippocampus (dHC). Early ethanol exposure is reported to alter the composition and function of forebrain NMDARs, well into adulthood, which could contribute to ethanol-induced TFC deficits. Data also suggests that reduced CS-mediated freezing in FAS rats trained with the 15 sec trace interval moderately correlates with diminished expression of phosphorylated extracellular signal-regulated kinase (pERK)—a protein engaged downstream of NMDARs that contributes to the maintenance of associative synaptic plasticity. Importantly, these results are not restricted to associative conditioning, as there are clear attentional and working memory components to the learning task, which are impaired in FAS individuals.

Introduction

Drinking while pregnant is detrimental to the ongoing development of the fetus. Fetal Alcohol Spectrum Disorder (FASD) covers the large variety of physical and mental deficits associated with *in utero* exposure to ethanol (Barr & Streissguth, 2001) and is considered the most common form of preventable mental retardation (Stratton et al., 1996). The severity of the deficit is dependent on the timing of alcohol consumption and the resulting peak blood alcohol concentration (BAC) (Hannigan 1996). Fetal Alcohol Syndrome (FAS) is the most severe and most commonly diagnosed subset of FASD. The criterion for diagnosis includes facial dysmorphology, central nervous system (CNS) dysfunction, growth retardation and confirmation of maternal alcohol consumption during pregnancy (Smitherman, 1994). As suggested by a recent meta-analysis, the prevalence rate within the US is anywhere from 2 to 7 in 1,000 births (May et al., 2009). There is also a subset of children who suffer from alcohol-related neurodevelopmental disorder (ARND) that possess the neurological deficits but lack the physical abnormalities seen in FAS individuals. FASD patients display a variety of cognitive impairments including those in attention, informational processing (Nanson and Hiscock, 1990; Lee et al., 2004) and executive functioning (Kodituwakku et. al, 1995; Roebuck et. al., 1999). These and other ethanol-related deficits are expressed early in development and persist well into adulthood (Streissguth, et al., 1994). Research tends to emphasize early or *in utero* treatments meant to prevent symptomatology later in life (Premji, Benzies, Serrett & Hayden, 2006; Tiwari & Chopra, 2011). Unfortunately, preventative treatment is difficult to implement since FASD is generally diagnosed after birth. Therefore, current research must investigate the underlying mechanisms affecting FASD individuals throughout the lifespan to improve current treatment

options. The primary focus of this thesis is directed towards adult learning and memory impairments that rely on attention and working memory.

Human studies of FASD have shown a decrease in overall brain volume as well as reductions in cerebral white matter (Archibald et al., 2001). Structural imaging studies show certain areas, such as the cerebellum, amygdala and prefrontal cortex (PFC), are decreased in volume due to fetal alcohol exposure (Bookstien et. al, 2001; Spadoni, McGee, Fryer et al., 2007; Coles et al, 2002), whereas effects on the hippocampus have been somewhat controversial. Some studies have reported that FASD patients have an underdeveloped hippocampus relative to control subjects (Bhatara et al., 2002) while others report that the hippocampus is unaffected by early ethanol exposure (Archibald et al., 2001). Despite the discrepancies in neuroimaging studies, it is well-accepted that those diagnosed with FASD are impaired in a variety of hippocampal-dependent learning tasks such as spatial learning, (McClelland and Goddard, 1996), verbal recall (Mattson et al., 1996) and spatial recall (Uecker and Nadel, 1998).

Rat models of FASD allow researchers to gain further insight by using methodologies that are not possible within the human population. Similar to human research, rat models have determined that the cerebellum, hippocampus (Onley et al., 2002) and PFC (Livy et al., 2003; Mihalick et al., 2001) undergo ethanol-induced excitotoxicity. The current study examines the effects of ethanol administration across postnatal days (PD) 4-9, a period that corresponds to the third-trimester in humans in which the brain is undergoing a rapid growth spurt (Bayer et al., 1993; Gil-Mohapel et al., 2010). Postnatal binge-like ethanol exposure in rat pups across PD 4-9 contributes to a variety of learning deficits seen in spatial, contextual, associative and executive functioning tasks. (Goodlett & Peterson, 1995; Hunt, Jacobson & Torok 2009; Hamilton et al., 2011). Past research indicates that ethanol-induced learning deficits may persist throughout

adulthood in rats, similar to humans (Johnson & Goodlett 2002). The current study, therefore, measures cognitive performance of adult FAS rats during a Pavlovian fear conditioning task, which is dependent on PFC-amygdala-hippocampal circuitry (Pitkänen et al., 2000).

Trace fear conditioning is a challenging form of Pavlovian conditioning in which a neutral conditioned stimulus (CS), a tone, is associated with an aversive unconditioned stimulus (US), a foot shock, to elicit a conditioned fear response (CR), freezing. Trace fear conditioning is unique in that there is a stimulus-free period separating CS-offset from US-onset. This duration of time is referred to as the trace interval. Adolescent FAS rats show impairment in trace but not delay fear conditioning, in which the CS and US overlap and co-terminate (Wagner & Hunt, 2006). Our lab has recently replicated and extended these findings in adult FAS rats trained with a 30 sec trace interval (data not shown). The data revealed a dose-dependent impairment such that the rats who received the highest concentration of ethanol performed the worst (i.e. showed the lowest level of freezing during CS tone presentations). It remains unclear, however, whether the observed deficit is due to the duration of the trace interval or the interval separating CS-onset from US-onset, known as the inter-stimulus interval (ISI).

As FASD research moves forward, it is important to distinguish and identify behavioral deficits specific to this population. In order to properly use trace fear conditioning for FAS-based research, it must first be determined whether or not the ethanol-induced deficits are specific to the trace interval, independent of the ISI duration. For the purpose of this study, rats were trained with either a short (5 sec) or long (15 sec) trace interval, while keeping the ISI constant at 20 sec. As the trace interval lengthens, trace fear conditioning becomes increasingly difficult to acquire, even for control rats (Gallistel & Gibbon, 2000). During the trace interval, subjects must maintain a memory trace of the neutral tone CS in order to associate it with the

aversive US (Pavlov, 1927). The current study is meant to demonstrate that FAS deficits observed in rats are due to the presence and length of the trace interval, independent of the ISI. If correct, future research may manipulate the trace interval while leaving the CS duration constant (thereby altering the ISI).

The medial prefrontal cortex (mPFC) is engaged during trace fear conditioning (Baeg et. al, 2001; Gilmartin &McEchron, 2005) and has been proposed to place predictive value on the CS (Guimarais et al., 2011). Furthermore, a subset of neurons within the mPFC synapse onto themselves and adjacent neurons through recurrent collaterals, allowing neurons to sustain firing across the trace interval and further encourage CS-US association (Wang, Stradtman, Wang & Gao, et al., 2008). Since neuronal activity occurs within the mPFC during the trace interval, the mPFC may undergo synaptic plasticity necessary for forming the CS-US association. (Gilmartin and McEchron, 2005). Temporary pharmacological inactivation of the mPFC suggests that this region is also involved in the storage of the CS-US memory. Even a month after trace fear conditioning, infusions of the γ-aminobutyric acid-A ($GABA_A$) agonist, muscimol, into the mPFC prior to testing reduces levels of conditioned fear to CS-alone presentations (Blum, Herbert & Dash, 2004).

Similar to the mPFC, CA3 of the dHC is also thought to contain recurrent collaterals capable of providing the positive feedback necessary for sustained spiking across the trace interval (Amaral & Witter 1995). For instance, Chowdhury et al., 2005 trace fear conditioned mice with variable (0 to 20 sec) trace intervals and then chemically lesioned the dHC post-training. Only mice trained with the longest trace interval showed impaired CS-mediated freezing. A similar study showed that the dHC is only engaged with trace intervals longer than approximately 10 secs (Guimarais et al 2011). Taken together, these studies suggest that the

mPFC and dHC may work in parallel to encode and store the memory trace during trace fear conditioning, with the hippocampus crucially involved with longer trace intervals.

In order to successfully acquire trace fear conditioning, glutamatergic synaptic plasticity is necessary within the mPFC and dHC (McLaughlin et al., 2002; Runyan, Moore & Dash, 2004). At excitatory glutamatergic synapses lie N-methyl-d-aspartate NMDA receptors (NMDAR) which are thought to play a key role in associative learning and memory. NMDARs are unique in that they require both glutamate binding and postsynaptic depolarization for the channel to open. The dual gating mechanism recognizes corresponding pre- and postsynaptic activity, which is essential for long term potentiation (LTP). LTP is characterized by the strengthening between synapses that occurs in response to associative learning (Brown et al, 2003).

LTP occurs in many regions of the brain, including the hippocampus, prefrontal cortex and amygdala (Lindquist & Brown, 2004; Malenka, 1994) which are all engaged during trace fear conditioning. In terms of FAS, ethanol exposure blocks NMDAR activity while enhancing $GABA_A$ receptor activity, leading to a compensatory increase in NMDARs at the postsynaptic terminal. Following the withdrawal of ethanol, NMDARs return to their normal activity level, but overexcite the neuron due to the upregulation of postsynaptic NMDARs. This over excitation of NMDAR function results in calcium-induced excitotoxicity within the brain resulting in cell loss as previously reported in ethanol-exposed humans and animals (Hendricson et al., 2007).

The subunit composition of NMDARs is also altered due to perinatal ethanol exposure, influencing the receptor's overall function (Livy et al., 2003; Mihalick et al., 2001). Since blocking LTP induction inhibits memory acquisition and consolidation (Bliss and Collingridge,

1993), it seems plausible that early ethanol exposure may alter the NMDARs engaged in and responsible for LTP. During LTP, downstream transduction cascades are triggered, inducing protein synthesis. Included in this cascade is the phosphorylation of extracellular signal-regulated kinases (pERK), which is heavily involved in the maintenance or expression of LTP (Peng et al., 2010). Past studies report that both pre- and post-training bilateral infusions of pERK inhibtitor U0126 in the mPFC create long-term memory deficits in trace fear conditioning. The same study then inhibited pERK within the hippocampus at varying intervals following trace fear conditioning. The results imply that pERK is expressed within the mPFC in conjunction with, or possibly even before, pERK expression in the hippocampus (Runyan, Moore & Dash, 2004). The current study uses pERK as a biomarker for NMDAR-dependent synaptic plasticity, which is predicted to show a positive correlation with ethanol-induced trace fear conditioning deficits.

In summary, FASD patients and rodent models show comparable behavioral deficits in a variety of learning and memory tasks. Specific forebrain areas that are targeted by ethanol exposure include the prefrontal cortex, amygdala and hippocampus, which are all involved in trace fear conditioning. FAS rats are predicted to have errors in acquiring or expressing previously learned associative fear when conditioned with a long (15 sec), but not short (5 sec), trace interval, due to the increased difficulty of the task. If this is true, one explanation may arise from decreased NMDAR function, which is necessary for the consolidation of trace fear memories through LTP. One way to look at NMDAR function and synaptic plasticity is by measuring levels of pERK expression. Protein expression in CA3 of the dHC is of particular interest due to the recurrent collaterals that potentially maintain spiking across longer trace intervals. Since the current study is already manipulating the duration of the trace interval, we

aim to verify the necessity of the dHC with longer but not shorter trace intervals via pERK cell counts. We therefore hypothesize that ethanol exposed rats will show impaired acquisition and consolidation of fear responses in trace fear conditioning with the 15 but not 5 sec trace interval. The predicted behavioral deficit should then correlate with NMDAR dysfunction, which would be seen as a decrease in pERK expression within the dHC, particularly within CA3. It is important to note that stained cells were only counted in rats trained with the long 15 sec trace interval as part of this thesis.

Materials and Methods

General information

Subjects

Sixty male and female Long Evans rats approximately 70 days old were used in the following experiment. Rats were bred within the Psychology Department vivarium by pairing a single male and female for one week. Beginning 21 days after their first day of coupling, the female dam was checked twice a day to monitor birth. The pups were weaned on postnatal day (PD) 21, same sex housed with 2-3 same-sex littermates per cage and then placed into individual cages at PD 60. Every rat was provided with ad libitum access to food and water. The colony room was on a 12h light-dark cycle (lights out 1800h). All procedures were conducted in concordance with The Ohio State University's Institutional Animal Care and Use Committee (IACUC).

Neonatal treatment

Within each litter, the pups were sorted into three neonatal treatment groups: unintubated control (UC), sham intubated control (SI), and an ethanol exposed group (5E) that received an ethanol-milk solution of 5g/kg/day, containing 11.33% ethanol by volume. Between PD 4-9, the 5E pups

were administered the ethanol-milk solution by intragastric intubation twice daily, 2 hr apart as proposed by Goodlett & Johnson (1997), along with a third intubation of pure formula milk. The SI group underwent the same intubation process as the 5E group, but did not receive any solution.

BAC (blood alcohol content)

The SI and 5E groups received a tail clip procedure after the second intubation on PD 4. Approximately 20μL of blood was collected in a capillary tube from all pups within both groups. Blood samples from the SI rats were discarded whereas blood samples from the 5E rats were transferred into microcentrifuge tubes and then centrifuged for ten minutes. The plasma was extracted and later analyzed for peak BAC using an Analox GL5 Analyzer (Analox Instruments, Lenenberg, MA). The instrument was able to assess ethanol levels by determining the amount of ethanol-induced oxygen depletion within the plasma sample.

Behavioral Procedures

Design

Three neonatal treatment groups were given a single session of trace fear conditioning (5 or 15 sec trace interval). Over the following two days, all subjects were submitted to a context and tone test, performed in counter-balanced order across animals. A separate group of SI and 5E pups received a single session of trace fear conditioning (15 sec trace interval) and were euthanized an hour later for immunohistochemical (IHC) analysis.

Trace Fear Conditioning

All rats underwent the same training and testing procedures. Twenty-three rats were trained with the 5 sec trace interval (UC, n = 8; SI, n = 8; 5E, n = 7) and twenty-five were trained with the 15 sec trace interval (UC, n = 9; SI, n = 8; 5E, n = 8). Animals were run two at a time in separate operant chambers (Coulburn Instruments, Allentown, PA). During trace fear conditioning, each rat was transported in its' home cage, carried two at a time, one on top of the other, by the experimenter and brought into a well-lit room. After wiping the chambers down with a vinegar-water solution, animals were placed into the darkened chamber with metal grid bars on the floor. Trace fear conditioning consisted of ten paired tone-foot shock presentations. The neutral CS was a 2.8 kHz, 80 dB tone presented through a speaker located within each chamber whereas the aversive US was a 1000 ms, 0.8 mA scrambled foot shock administered by the grid bar floor with an inter-trial interval (ITI) of 240 ± 30 sec. Rats were trained with either a 5 or 15 sec trace interval separating the tone CS offset and foot shock US onset. In order to keep the ISI constant, rats trained with a 5 sec trace interval had a 15 sec tone presentation, whereas the rats with a 15 sec trace interval had a 5 sec tone presentation. The rats were taken out of the chamber approximately 2 min after the last shock presentation. Twenty-four hours later, the rats underwent either tone or context testing, which was counterbalanced across animals.

Context Test

The rats were brought into the conditioning room as described above for the conditioning phase and placed into the chambers for 14 min. The purpose of the context test was to measure contextual fear without the presence of the CS tone. Freezing was assessed across 2 min bins between 2-12 min of contextual exposure.

Tone Test

In order to measure the conditioned fear associated with the CS, rats were tested with tone presentations in a novel context, including transportation cues. Therefore, the rats were brought into the room on a metal wheeled cart while remaining in their home cages that were covered with a towel. The testing room was also dark with only a red light on, which the rats cannot sense. To change the appearance of the chamber, it was scented with Windex®, illuminated by a hanging light, had a distinguishable laminated pink figure attached to the front door and the grid bars were covered with a sheet of plexiglass. The first tone was played approximately 2 min after the rats were placed into their chambers. Four 2 min tones were presented, separated by a 1 min interval between each tone. Freezing was quantified during the 2 min duration of each tone presentation.

Freezing analysis

Freezing in rats is defined as no movement apart from respiration (Blancherd & Blancherd, 1969). The rate of freezing was determined by using FreezeScan, which is an automated system that detects movement above a certain threshold.

Immunohistochemistry

Twelve rats were sacrificed approximately 60 min after trace fear conditioning in order to measure the expression of phosphorylated ERK in CA3 of the dorsal hippocampus. Animals were euthanized with 200 mg/kg of Euthasol® and then immediately perfused with 0.9% saline followed by 4% paraformaldehyde. The brain was then extracted, weighed and fixed in 4%

paraformaldehyde for 48 hours. The entire dHC was sectioned with a thickness of 50μm by a vibratome.

Every sixth section was used for staining, starting from the beginning of the hippocampus and finishing close to the convergence of the dorsal and ventral hippocampus, resulting in 5-6 sections per subject. Selected sections were rinsed with TBSt, and then submerged in a sodium citrate buffer while heated in a steam bath for 20 min at 37^{O} C. Sections were rinsed and then quenched for 1 hr with 0.3% H_2O_2 in methanol. The tissue was then rinsed before blocking in 5% normal goat serum (NGS) for 1 hr. Tissues were submerged in a 1:100 dilution of pERK (9102L; Cell Signaling, Danvers, MA) and left to incubate over night. The next day, sections were rinsed with TBSt and then incubated in a 1:200 dilution of anti-rabbit secondary anti-body (BA-1000; Vector, Burlingame, CA) for 90 min. The sections were incubated in a DAB-ABC kit for approximately 70 sec, when the desired level of staining had been achieved. Sections were rinsed again, this time with 10M PBS, before being mounted on subbed slides. The slides were left to dry overnight and then dehydrated in 70%, 95% and 100% ethanol for 2 min each, followed by 5 min in xylene and then coverslipped using permount.

Stain Quantification

Images were taken at 10 x magnification and stitched together to form a larger cohesive image using NIS Elements. Regions of Interest (ROI) were manually drawn using Image J (National Institute of Health, Bethesda, MD) to outline, separate and provide the area for the subregions of interest within the dHC. The initial analysis focused on the CA3, which was then further divided into the CA3ab and CA3c subregions (Figure 5). Image J was also used to manually count cells exhibiting pERK expression. Animals were assigned randomized identification numbers so that

pERK expression could be examined without bias. Cell numbers were calculated based on a cell-to-area ratio accounting for differences in area (mm^2) across slices.

Statistics

Behavioral and IHC data was analyzed using mixed-design ANOVAs, including repeated measures analyses when necessary. A significant post-hoc (Fisher's PLSD) effect indicates $p < 0.05$.

Results

Body Weights

Body weights for all sixty rats (behavior and IHC) were recorded across PD 4, PD 9, PD 15, PD 21, PD 30, PD 45 and PD 60. A one-way (treatment) ANOVA showed statistically significant differences in weights between treatment groups for PD9 $F(2, 61) = 15.68$, $p < 0.005$, PD15 $F(2, 61) = 10.07$, $p < 0.005$, PD 21 $F(2, 61) = 6.45$, $p < 0.005$ and PD30 $F(2, 61) = 5.76$, $p = 0.005$. There were no significant differences in weights across groups for PD4 ($p = 0.28$), PD45 ($p = 0.11$) or PD60 ($p = 0.17$). Although there was a significant difference in body weight between 5E and control rats early in development and adolescence, 5E rats had body weights similar to SI and UC rats by the time trace fear conditioning was initiated (see Table 1), eliminating a body weight confound.

Freezing

Conditioning

Initially, the post shock freezing data was analyzed with a mixed design 3 (Treatment Group) X 2 (Trace Interval) X 10 (Trials) repeated measures ANOVA. Results indicate that neither

between-subject main effects nor their interaction reached significance, whereas the within-subject factor, trial, did reach significance $F(9,378) = 6.55$, $p < 0.0001$ (Figure 2). Post shock freezing is considered a fear CR and is thought to be predictive of the aversive US (Fanselow, 1980). The results indicate conditioned fear (freezing) increased as a function of trial number, but did not significantly vary across training group or trace interval.

The conditioning data was further analyzed separately for each trace interval (5 sec and 15 sec) using multiple 3 (treatment) x 10 (trial) repeated measures ANOVAs. Freezing rates were assessed during each of the ten tone CS presentations as well as across the trace interval prior to US onset. With the 5 sec interval, the repeated measures ANOVA for tone-elicited freezing did reveal a significant main effect for treatment group, $F (1, 171) = 4.56$, $p < 0.05$ (Figure 3). Post-hoc analysis showed a statistically significant difference between UC and 5E, as well as SI and 5E rats. It is important to note, however, that the 5E rats actually display significantly higher freezing compared to SI and UC groups. In terms of freezing across the trace interval, the analysis did not reach statistical significance for the treatment group main effect ($p = 0.19$).

With the 15 sec interval, the 3 x 10 repeated measures ANOVA applied to freezing behavior across the 10 tone presentations, and the trace interval, revealed a statistically significant main effect, $F(2, 180) = 4.82$, $p < 0.05$ and $F(2, 180) = 11.88$, $p < 0.0005$, respectively (Figure 4). Post-hoc analyses revealed significant differences between UC and 5E rats, with higher levels of freezing seen in the UC subjects relative to 5E, for both measures. In addition, a post-hoc analysis for trace interval freezing revealed significantly higher levels of freezing for UC than SI rats.

Taken together, the data suggests that there is no difference between treatment groups or trace intervals when freezing is analyzed 1 min post shock (Figure 2). When trained with a 5 sec trace interval, FAS rats are able to acquire trace fear conditioning comparable to control rats (Figure 3), whereas those trained with a 15 sec interval might have acquisition deficits relative to UC rats (Figure 4). Post shock freezing is typically the measure used to indicate the subject's rate of learning (Lindquist, Mahoney, Steinmetz, 2010). Results assessing freezing during tone presentations or across the trace interval must be interpreted somewhat more cautiously.

Testing

Freezing behavior for the context and tone tests were independently analyzed by repeated measures ANOVAs, with two between subject factors (treatment and trace interval) and one within-subject factor. The repeated measures (within-subject) factor was five 2 min bins for context testing and four 2 min tone presentations for the tone test. For freezing during the context test, neither between subject factors reached significance, though there was a significant repeated measures effect $F(4, 168) = 4.56$, $p < 0.01$, indicating that freezing decreased across exposure time (data not shown). For the tone test, there was a significant main effect for treatment group $F(2, 126) = 3.23$, $p < 0.05$ and trace interval $F(1,126) = 8.04$, $p < 0.001$, but not their interaction (data not shown). As above, there was a significant repeated measures effect across tone presentations, $F(3, 126) = 10.36$, $p < 0.001$ with freezing decreasing across consecutive tone presentations.

Repeated measure analyses applied to rats conditioned with the 5 sec trace interval did not show statistically significant differences in freezing between treatment groups during the context ($p = 0.79$) or tone test ($p = 0.44$). Prior to the first tone presentation, all groups of rats

froze at similarly low levels ($p = 0.88$), indicating a successful context shift across groups (Figure 3).

Rats conditioned with the 15 sec interval, also showed no significant differences between treatment groups for contextual freezing ($p = 0.22$). For the tone test, there were also no significant group differences in freezing behavior prior to the first tone presentation ($p = 0.27$), suggesting again that the context shift was successful. Freezing to the tone presentations during the tone test approached, but just missed significance, $F(2,66) = 3.41$, $p = 0.05$. (Figure 4) Nevertheless, post-hoc analyses indicated that UC rats froze significantly more than 5E rats, a result consistent with previous results from the Lindquist lab.

Cell Counts

pERK stained cells in the CA3 region of the dorsal hippocampus from six SI and six 5E rats were counted and quantified. Multiple one-way (treatment) ANOVAs were applied to CA3ab, CA3c, or the entire CA3 region. Results revealed a statistically significant main treatment group effect for CA3ab, $F(1,140) = 13.25$, $p < 0.0001$ and for the entire CA3 region, $F(1,140) = 6.79$, $p < 0.05$. The treatment group effect for region CA3c was not statistically significant, $p = 0.31$ (Figure 5). Results indicate the pERK expression was lower in 5E rats compared to SI controls.

A Pearson's r correlation was measured between behavioral subjects and IHC subjects trained with a 15 sec trace interval (SI and 5E only), yielding a moderate correlation between freezing and pERK expression within CA3ab ($r= 0.39$) and the entire CA3 ($r^2= 0.42$; data not shown).

Discussion:

The current results support the hypothesis that the trace interval duration is primarily responsible for the ethanol-induced deficits in CS-mediated freezing following trace fear conditioning, independent of the conditioning ISI. During trace fear conditioning, FAS rats trained with a 5 sec trace interval displayed freezing rates comparable to control rats when freezing was measured across the trace interval for each trial and even froze significantly more than UC and SI rats when assessed during tone presentations (Figure 3). 5E rats trained with a 5 sec trace interval also displayed freezing levels equivalent to UC and SI rats during both tone and context tests (Figure 3).

However, when conditioned with a 15 sec trace interval, 5E rats have difficulty acquiring the task relative to UC rats (Figure 4). Although somewhat unclear, SI rats show significantly less freezing compared to UC rats, when freezing is assessed during tone presentations, suggesting a possible acquisition error in SI rats as well (Figure 4). It is important to note, however, that SI rats only show deficits across a single measurement of acquisition, proposing only a mild deficit, if that. As expected, FAS rats display significantly lower freezing percentages during the tone test relative to control rats, but have no difficulty recognizing and freezing to the conditioning context (Figure 4). Contextual freezing rates suggest 5E animals trained with a 15 sec trace interval are able to express previously acquired fear. Although the hippocampus is a key component in contextual conditioning (Smith & Mizumori 2006), 5E rats seem to learn comparable to controls. The 15 sec trace interval-specific deficit within FAS rats suggests their learning and memory deficits rely on the difficulty of the task, not just the task's dependence on the hippocampus.

IHC analyses for rats trained with a 15 sec trace interval, showed a significant reduction in pERK expression within the entire CA3, and specifically, the CA3ab (Figure 5) subregion of the dHC in ethanol-exposed compared to ethanol-naïve rats. Interestingly, there was no significant difference between the mean areas of CA3, CA3c or CA3ab subregions of the hippocampus between treatment groups (data not shown). Thus, early ethanol exposure does not appear to restrict hippocampal growth, though we cannot rule out possible reductions in cell numbers. Analysis of the behavioral data for the twelve rats used for IHC did not reveal any significant differences in freezing percentages between the SI or 5E treatment groups during trace fear conditioning (data not shown). Therefore, it is currently uncertain whether the behavioral deficits are due to an inability to acquire the CS-US association leading to less consolidation, or if the deficit is solely dependent on errors in consolidation.

It has been previously discovered that rats who receive postnatal ethanol are impaired at trace, but not delay fear conditioning (Wagner & Hunt 2006). Since only the 5E rats trained with a 15 sec, but not 5 sec, trace interval exhibit trace fear conditioning deficits relative to the control groups (Figure 2), our data suggests that ethanol-exposed rats can, in fact, learn trace fear conditioning when there is a short trace interval. Interestingly, there was an acquisition deficit in both the SI and 5E groups trained with a 15 sec interval, relative to UC control rats. However, SI rats have shown freezing levels comparable to UC rats during the tone test following conditioning (Figure 4). It is also important to note that there was no significant difference found between 5E rats and control rats when freezing was assessed across post shock intervals (Figure 2), which is known to be highly correlated with acquisition. During the context test, all groups showed significant freezing to the context, suggesting that FAS rats are able to express previously acquired contextual fear (Figures 3 and 4)

The dHC has been suggested to be involved with trace fear conditioning when rats are conditioned with long, but not short trace intervals (Guimarais et al., 2011). The current thesis manipulated the trace interval length in order to determine its' significance in ethanol-induced trace fear conditioning deficits (Figure 5). The behavioral data indicates that 5E rats trained with a 15 sec trace interval had potential difficulties acquiring the CS-US association (Figure 4). IHC analyses confirm a consolidation error, although there was no visible behavioral acquisition deficit within this specific subset of 5E rats, compared to SI rats. Inconsistency across groups (behavioral and IHC) calls for a greater focus on ethanol-induced acquisition deficits in later studies. That is, can the FAS animals learn normally but are impaired at storing or consolidating the CS-US association or is there an acquisition deficit responsible for the low levels of consolidation (as seen with pERK)? More research will be required to dissociate this issue.

Past FASD research examined hippocampal cortical cultures 1 hr following postnatal ethanol exposure (PD5 or PD21) and showed a significant decrease in pERK activity (Chandler & Sutton 2005). Ethanol has also been demonstrated to decrease the expression of brain derived neurotrophic factor (BDNF)-activated pERK expression within the CA1 of the hippocampus (Davis et al., 1999). Accordingly, the current results show a significant reduction of pERK expression within adult FAS rats compared to SI rats (Figure 5), which is thought to affect memory consolidation, but not acquisition or retention, of trace fear condition (Villarreal & Barea-Rodriguez 2006). pERK expression was examined within the CA3 subregion of the dHC which contains recurrent collaterals, which may be necessary to create a memory trace by maintaining spiking across the trace interval in order to associate the CS with the US (Rodriguez and Levy, 2001).

Although the dHC has been the primary focus of the current experiment, there are other brain regions that play an important role in trace fear conditioning. For example, the mPFC also contains recurrent collaterals that are thought to be activated across the trace interval (Wang, Stradtman, Wang & Gao, 2008). The current data then supports the idea that the spiking maintained by the mPFC's recurrent collaterals is sufficient for the acquisition and expression of delay and trace fear conditioning with a short trace interval. However, it seems that when the trace interval is lengthened and the task becomes more difficult, the mPFC is no longer sufficient to maintain spiking, and requires the assistance of the dHC. When the recurrent collaterals of the dHC are not functioning optimally, as in the FAS rats, the subject is not able to express the previously acquired fear response to the tone-alone presentations, if conditioned with a longer trace interval.

Furthermore, the mPFC and ventral hippocampus (vHC) directly synapse on the basolateral amygdala (BLA), which is responsible for the expression of previously acquired trace fear conditioning (LeDoux 2000). The dHC must communicate to the BLA via the vHC, and possibly the mPFC, since the dHC and BLA are not directly connected (Sierra-Mercado et al., 2011). Therefore, the overall role of the dHC in trace fear conditioning is still unclear. When pre-training APV (a NMDAR antagonist) injections are administered within the dHC, animals are able to acquire the task, even with a long trace interval (28 sec). However, when the APV is administered prior to testing, so that the dHC is unaffected during conditioning, the rats are unable to express previously acquired conditioned fear (Czerniawski, Ree, Chia & Otto, 2011). The data then suggests that if NMDARs within the dHC are not functioning during trace fear conditioning then other brain regions are able to compensate for its' lack of activity and the task is acquired. However, if the dHC is functioning during conditioning, then learning occurs in the

dHC and is necessary for the expression of the previously learned conditioned fear response. To date, however, there is no certainty as to which brain region, (dHC, vHC, or mPFC) or combination of regions, is ultimately driving the freezing fear response to the BLA. The current results are expected to help clarify the role of the dHC when using different trace intervals.

Future studies are needed to further dissociate the specific roles of the dHC, vHC and mPFC in trace fear conditioning. The current thesis is only part of a larger study in which the expression of pERK within the CA1 and dentate gyrus (DG) of the dHC will also be examined. In addition, the brains from rats trained with a 5 sec trace interval will be stained for pERK in the dHC 1 hr following trace fear conditioning, in addition to a UC "sit" group. The analysis of pERK expression within the 5 sec trace interval group will also be divided between regions CA1, CA3ab, CA3 and DG of the dHC. Examining the changes in pERK expression in the 5 sec trace groups will also help to further explain the dHC's role with varying durations of the trace interval.

Although somewhat speculative, we hypothesize that decreased expression of pERK could reflect reduced LTP due to early ethanol exposure. NMDARs are tetramers composed of two NR1 and two NR2 subunits. The NR2 subunits, either NR2A or NR2B receptors, differ in kinetics so that the NR2A receptor is more easily activated, opening and closing more rapidly than the NR2B receptor. Early in development, NR2B is more widely expressed within the brain, whereas NR2A becomes more expressed across development (Monyer et al., 1994; Okabe et al., 1998). Ethanol exposure enhances $GABA_A$ receptor activity and inhibits NMDAR activity. Once ethanol is no longer present, there is a drastic increase in glutamatergic activity (Hendricson et al., 2007). Following the withdrawal of ethanol, the presence of NR2B is highly detrimental to the survival of neurons by allowing a lethal amount of calcium into the cell,

causing excitotoxicity. As a neuroprotective strategy, the neurons begin to insert more NR2A receptors into the postsynaptic terminal (Nixon et al., 2002; Nixon et al., 2004; Lovinger, 1995). With NR2A's faster kinetics, it makes it more unlikely for ethanol-induced excitotoxicity to occur. In fact NR2A receptors allow half the amount of calcium influx compared to NR2B receptors (Erreger et al., 2005; Sobczyk and Svoboda, 2007). However, the compensatory reaction of inserting more NR2A receptors in postnatal rats might have a negative effect on learning and memory across the lifespan. The shift in a greater NR2A/NR2B ratio has been loosely suggested to affect learning-related synaptic plasticity by increasing the threshold for LTP induction. In other words, ethanol-induced change in NMDAR composition could make it more difficult for FAS rats to induce the LTP necessary for forming the CS-US association (Abraham, 2008). The ethanol-induced effects on NMDAR composition will also become clearer as the overall study continues past the scope of the current thesis.

In support of the postulated alterations in NMDAR composition, preliminary data from the Lindquist Lab suggests that D-cycloserine (DCS), a competitive NMDAR partial agonist, increases performance of FAS rats during a dHC-dependent behavioral learning and memory task known as the context pre-exposure facilitation effect (CPFE) (Matus-Amat, Higgins, Barrlentos & Rudy, 2004). DCS binds at the NMDARs secondary glycine binding site found on the NR1 subunit of the NMDAR and in the case of FAS rats, could potentially decrease the threshold required to induce LTP, promoting more robust learning and memory.

Although this data is only preliminary, it does suggest possible pharmaceutical treatment for adults with FASD. Although trace fear conditioning might seem like a specific paradigm, there are both attention and working memory components to this task as well. In another similar Pavlovian fear conditioning task, known as eyeblink conditioning, the participant must associate

a tone CS with an airpuff US to the eye, to elicit an eyeblink CR. When tested in humans, it has been found that the task cannot be acquired without the participant's conscious awareness of the tone's predicative value of the airpuff (Lovibond, Liu, Weidemann & Mitchell, 2011). Awareness of the CS-US association could potentially be true with trace fear conditioning as well, although it has not yet been examined. Not surprisingly, FASD patients are known to have deficits in attention, executive functioning, visual-spatial functioning, and learning and memory, all of which require a functional hippocampus (review: Alfonso-Leoches & Guerri, 2011). Although information is becoming more available to pregnant women, FASD will most likely continue to occur. Thus, it is important to continue research in this area in order to ameliorate the long-lasting deficits associated with this disorder.

Mean Body Weight (g)							
Treatment	PD 4	PD 9	PD 15	PD 21	PD 30	PD 45	PD 60
UC	10.2 ± 0.3	19.2 ± 0.8	30.8 ± 1.2	49.9 ± 2.1	98.9 ± 3.5	197.4 ± 8.2	280.7 ± 16.1
SI	9.9 ± 0.3	18.1 ± 0.5	29.0 ± 0.7	45.5 ± 1.5	91.1 ± 2.3	185.9 ± 6.3	268.2 ± 62.4
5E	9.5 ± 0.2	*15.0 ± 0.4	*25.4 ± 0.7	*41.8 ± 1.3	91.3 ± 2.3	176.4 ± 6.0	244.5 ±56.6

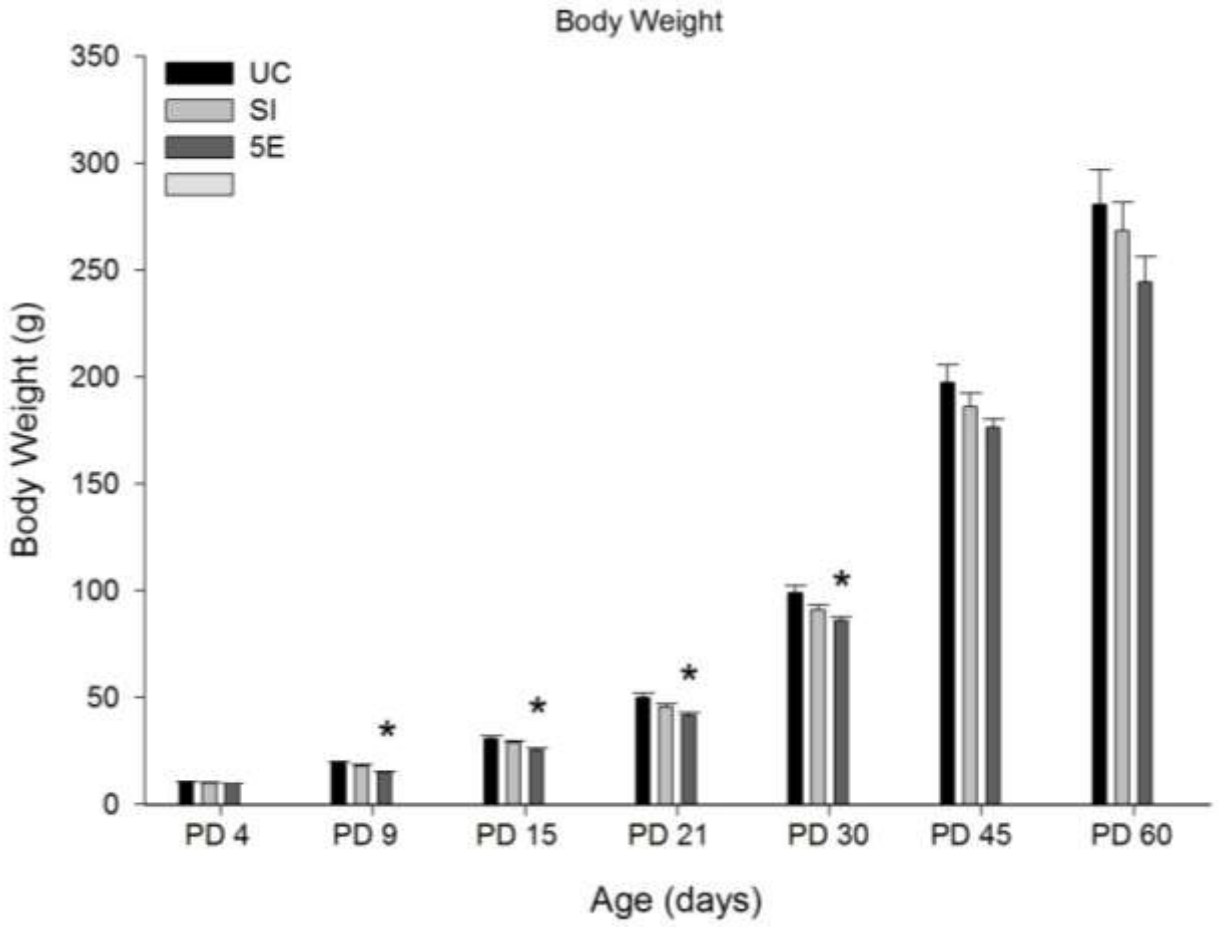

Figure 1. All rats were weighed periodically across development to monitor body weights. Although 5E rats weighed less than control rats for multiple weeks following the intubation period, all rats had comparable weights when tested. Both the table and graph show means and standard errors of all treatment groups across development. Astrics (*) indicate statistical significance between treatment groups.

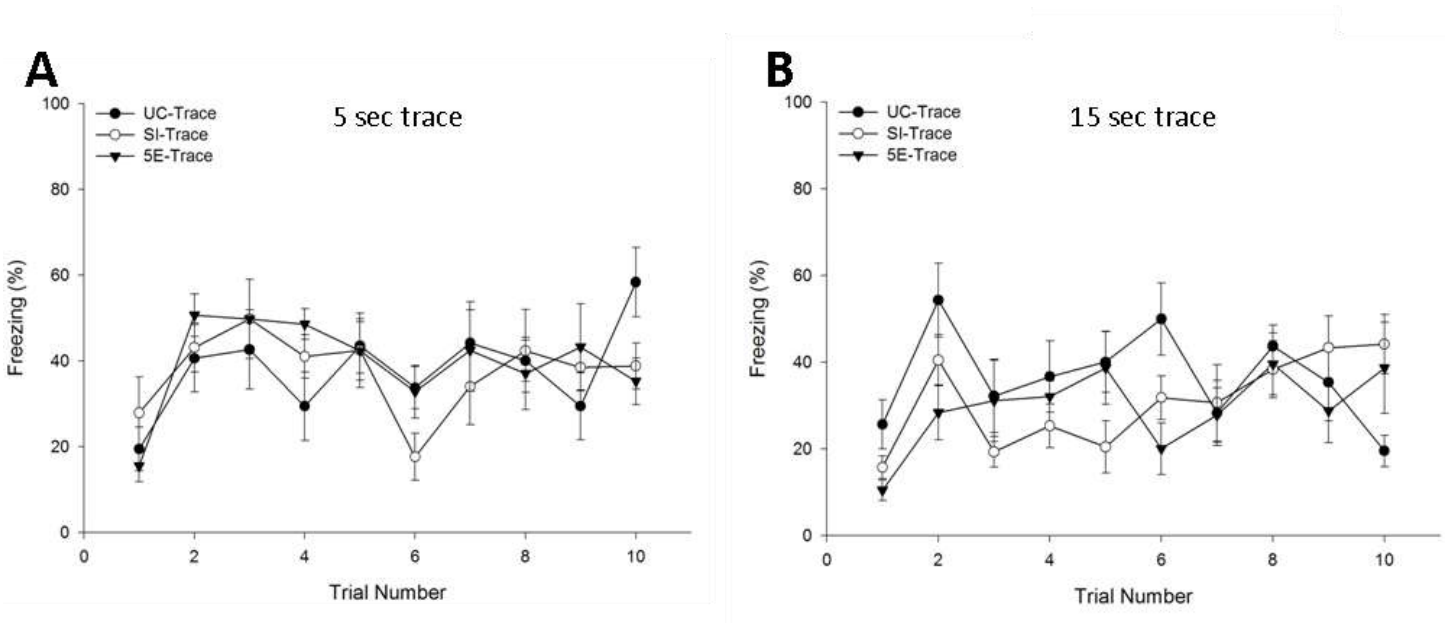

Figure 2. Freezing percentages were measured between trace interval groups during trace fear conditioning 1 min following foot shock presentations. A mixed measures ANOVA reveals that there was no significant difference in freezing percentages in rats trained with (A) a 5 sec trace interval compared to (B) those trained with a 15 sec trace interval, regardless of treatment group.

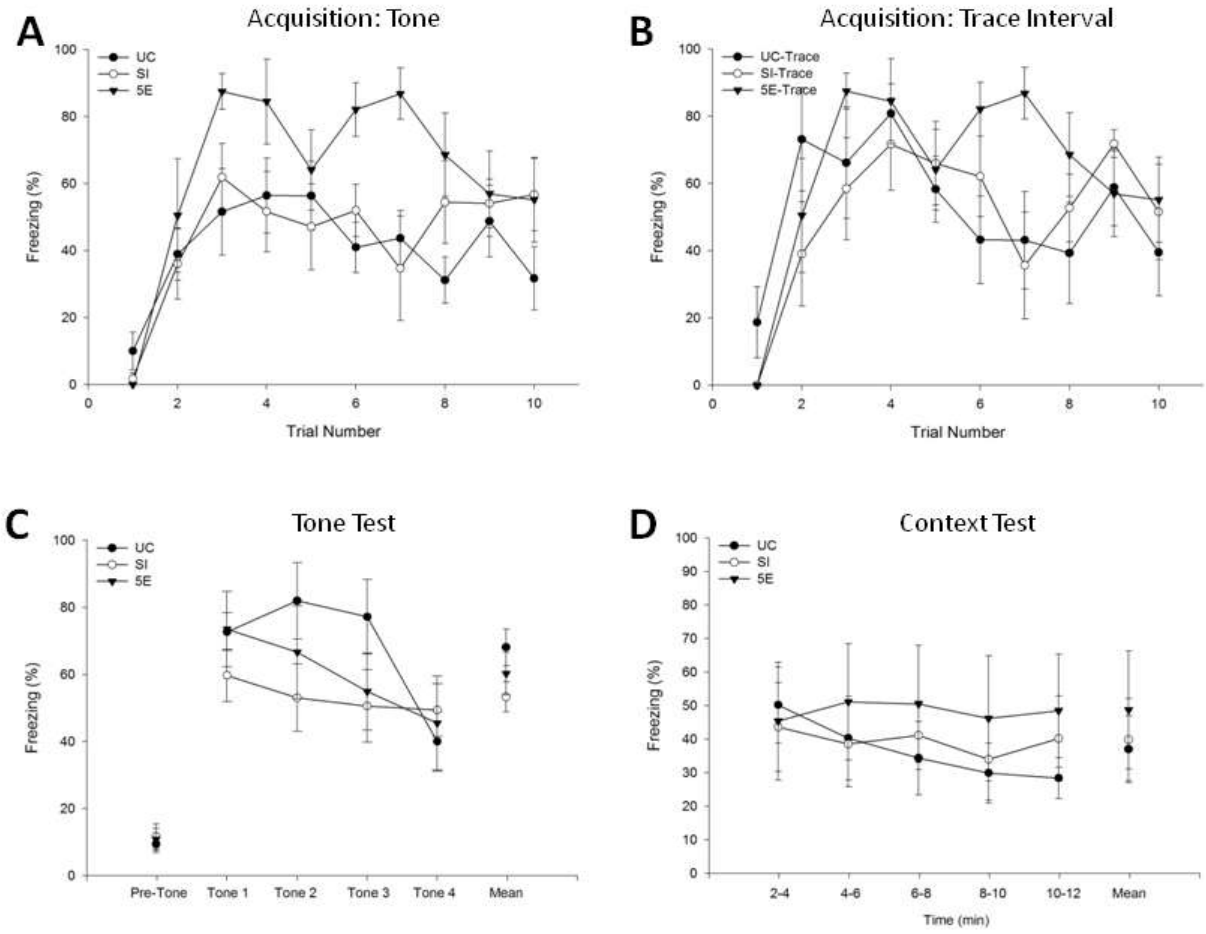

Figure 3. Freezing percentages were measured for all rats conditioned with a 5 sec trace interval during conditioning and testing. (A) Percent freezing (mean ± SE) for each tone presentation during trace fear conditioning for all treatment groups. (B) Percent freezing (mean ±SE) for each trace interval during trace fear conditioning for all treatment groups (C) Percent freezing (mean ± SE) for 1 min prior to tone presentations, each tone presentation and mean freezing levels during the tone test for all treatment groups. (D) Percent freezing (mean ± SE) in two minute bins from minutes 2 – 12 and mean freezing during the context test for all treatment groups.

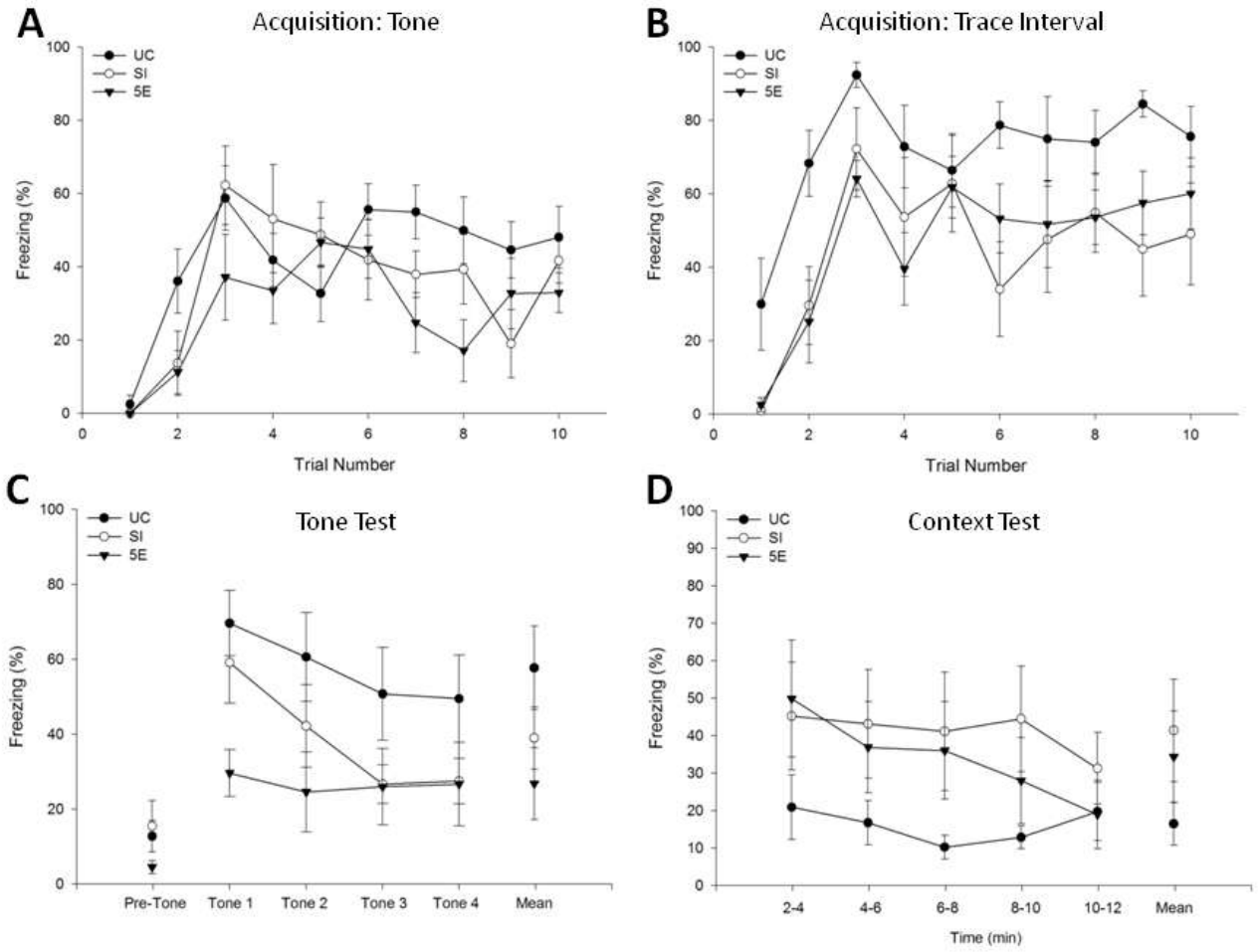

Figure 4. Freezing percentages were measured for all rats conditioned with a 15 sec trace interval during conditioning and testing. (A) Percent freezing (mean ± SE) for each tone presentation during trace fear conditioning for all treatment groups. (B) Percent freezing (mean ±SE) for each trace interval during trace fear conditioning for all treatment groups (C) Percent freezing (mean ± SE) for 1 min prior to tone presentations, each tone presentation and mean freezing levels during the tone test for all treatment groups. (D) Percent freezing (mean ± SE) in two minute bins from minutes 2 – 12 and mean freezing during the context test for all treatment groups.

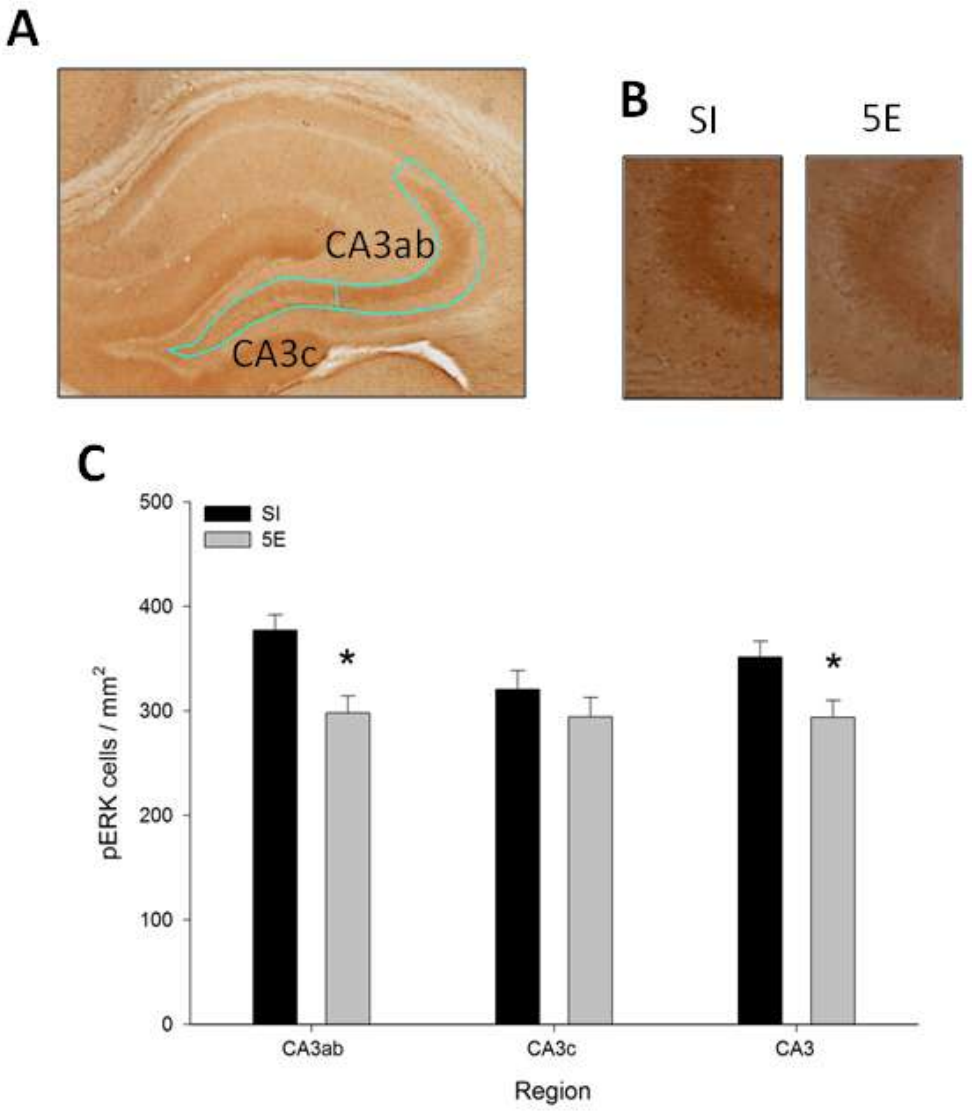

Figure 5. Immunohistochemical cell counts of pERK were used to quantify possible consolidation deficits within 5E rats relative to SI controls. (A) CA3 of the dHC was further broken down into CA3ab and CA3c subregions as illustrated. (B) Hippocampal sections are shown at 10 x magnification for both SI and 5E treatment groups. (C) pERK expression (mean ± SE) within CA3ab, CA3c and the entire CA3 region in rats trained with a 15 sec trace interval. Astrics (*) indicate a statistically significant difference between groups.

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